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Electron Microscopy of Intracellular Protozoa

Annual Report

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The distribution of circumsporozoite protein within developing P. malariae oocysts and salivary gland sporozoites and within P. cynomolgi exoerythrocytic stages was examined by immunoelectron microscopy using protein-A gold and monoclonal antibodies. Gold label was detected on the inner surface of peripheral vacuoles during oocyst maturation and the plasma membrane of the sporoblast. Salivary gland sporozoites and budding sporozoites in mature oocysts were labeled uniformly on the outer surface of their plasma membranes. The surface of sporozoites that ruptures into midgut epithelial cells were entirely covered with gold labels. Our results indicate that CS protein are synthesized in the oocyst before sporozoite formation and CS proteins of salivary gland P. malariae sporozoites are derived from the oocyst.

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19. Abstract (continued)

A mouse monoclonal antibody to the repeat regions of the P. cynomolgi CS protein densely labeled the plasma membrane and surface of 5-day old EE schizonts. Density of labeling decreased significantly as EE schizonts increased in size and maturity. Mature 8-day old schizonts with developing merozoites had little detectable labeling. Label was not observed in host cell cytoplasm or on the surface of infected hepatocytes. These findings indicate that epitopes associated with the repeat region of the P. cynomolgi CS proteins are sequestered within infected host cells during early EE development. Their absence from the host cell surface may make these epitopes poor targets for cellular or humoral immunity.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Localization of Circumsporozoite antigen in Exoerythrocytic Schizonts of *Plasmodium cynomolgi*

Abstract. We used colloidal gold probes and post-embedding immunoelectron microscopy to localize circumsporozoite (CS) antigen in 5- and 8-day-old *in vitro* cultures of *Plasmodium cynomolgi* exoerythrocytic (EE) schizonts. Both small uninucleate and large multinucleated EE schizonts were found in 5-day-old cultures. A mouse monoclonal antibody to the repeat region of the *P. cynomolgi* CS protein densely labeled the plasma membrane and surface of 5-day-old EE schizonts as well as the surrounding parasitophorous vacuole membrane and space. Density of labeling decreased significantly as EE schizonts increased in size and maturity. Labeling of large, multinucleated 5-day-old schizonts was sparse and limited to the surface of EE schizonts and to small patches of electron-dense material which were attached to the inner surface of the parasitophorous vacuole membrane. Mature 8-day-old EE schizonts with developing merozoites had little detectable labeling. CS antigen was not associated with internal structures within developing schizonts. Label was not observed in the host cell cytoplasm or on the surface of infected hepatocytes. These findings indicate that epitopes associated with the repeat region of the *P. cynomolgi* circumsporozoite protein are sequestered within infected host cells during early EE development. Their absence from the host cell surface may make these epitopes poor targets for cellular or humoral immunity.

Introduction

In vitro cultivation of exoerythrocytic (EE) stages of malarial parasites has only recently made this stage of the life cycle available for antigenic and immunological analysis. Most studies have used antibodies to specific sporozoite or erythrocytic-stage antigens as probes for determining the temporal expression and intracellular distribution of these molecules by immunofluorescence (IFA) or immunoperoxidase (IPA) antibody techniques. In general, exoerythrocytic schizonts appear to share antigenic determinants from both sporozoites and erythrocytic parasites and undergo a progressive loss of sporozoite-specific antigens as they mature. [1-5]

Persistance of circumsporozoite (CS) antigen in developing EE schizonts is of special interest because of its importance as a vaccine candidate. Studies by IFA and IPA techniques have shown that all or most of the CS protein is carried into hepatocytes by invading sporozoites where epitopes associated with the immunodominant repeat region persist on the surface of the growing schizont. [6-8] Studies of *P. berghei*, *P. falciparum*, and *P. vivax* have shown that CS antigen may persist throughout the complete EE cycle. [3, 5, 7] Thus, knowledge about the intracellular distribution of epitopes associated with the CS protein will be helpful in determining the potential effects of cellular and humoral immunity on developing EE parasites.

Millet et al. [9] described the *in vitro* cultivation of *P. cynomolgi* EE schizonts in primary cultures of rhesus (*Macaca mulatta*) hepatocytes. We used this *in vitro* system and monoclonal antibody specific for the repeat

region of the P. cynomolgi CS protein to localize this epitope in EE parasites and host cells. In contrast to studies of P. berghei, P. vivax, and P. falciparum, the immunodominant repeat region of the P. cynomolgi CS protein disappears as EE schizonts mature.

Materials and Methods

In Vitro Cultivation

Sporozoites of Plasmodium cynomolgi were obtained by feeding Anopheles dirus mosquitoes on an infected rhesus monkey. Fifteen days after the infective blood meal, mosquito salivary glands were dissected aseptically, resuspended into SMEM (Minimum Essential Medium supplemented with 10% fetal bovine serum, 2 gm/L bovine albumin, 10 mg/L insulin, 150 UI/ml penicillin, and 150 ug/ml streptomycin), and ground to release infective sporozoites.

Primary cultures of hepatocytes came from liver biopsy of a rhesus monkey. A liver fragment (approximately 2X2 cm) was collected by laparotomy and perfused with a collagenase-Hepes buffer solution to dissociate the hepatocytes which were then cultivated in SMEM as previously described. [9] One drop (25 ul) of the cell suspension, containing 3 X 10^4 hepatocytes, was deposited on each of four sites on the bottom of a 35 mm plastic tissue culture dish. Sedimentation, adherence, and resulting growth of the cells formed four disc-shaped (5 mm diameter) monolayers.

The day following hepatocyte dissociation, the drop of medium covering each monolayer disc was removed and replaced with 25 ul of medium containing 5×10^4 sporozoites. After 2 hours, the drops were removed and 1 ml of SMEM was added to each culture dish, then changed daily. Cultures were incubated for 5 to 8 days at $37^{o}C$, in an atmosphere of 5% CO_2 and 95% air.

Immunoelectron Microscopy

Monolayers of primary Rhesus hepatocytes which had been infected with P. cynomolgi sporozoites were carefully scraped from slides with a rubber policeman on days 5 and 8 postinfection. The cells were fixed for 60 min at room temperature in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, with 4% sucrose and washed three times by centrifugation with 0.05 M phosphate buffer with 4% sucrose. The samples were dehydrated at progressively lower temperatures between $4^{o}C$ and $-20^{o}C$ with 10-min-changes of 30%, 50%, 70% and 95% ethanol and transferred to a mixture of one part 95% ethanol - two parts LR Gold resin (London Resin Company) for 20 min. The cells were subsequently infiltrated at $-20^{o}C$ with three, one-hour-changes of LR Gold resin containing 0.75% (w/v) benzoin methyl ether as an ultraviolet initiator and left overnight in fresh resin. The samples were finally transferred to Beem capsules containing fresh resin, pelleted and polymerized at $-20^{o}C$ for 48 hours with an ultraviolet light. Ultrathin sections were cut with a diamond knife and collected on unsupported 300 mesh nickel grids.

Sections were etched for 30 min at room temperature in drops of a saturated aqueous solution of sodium metaperiodate, rinsed with distilled water and blocked for 30 min with a solution containing 5% nonfat dry milk,

0.9% NaCl and 0.01% Tween 20 in 0.1 M phosphate buffer, pH 7.3 (PBS-Milk-Tween). The sections were labeled in drops of mouse monoclonal 13E1 diluted to a final concentration of 2.5 ug/ml with a solution containing 1% bovine serum albumin (Fraction V) (ICN Immunobiologicals), 0.9% NaCl and 0.01% Tween 20 in 0.1 M phosphate buffer, pH 7.3 (PBS-BSA-Tween). The mouse monoclonal recognized the repeat region of the P. cynomolgi CS protein and was produced and characterized as described by Cochrane et al.[10] After a two hour incubation at room temperature, the grids were washed for 15 min in three changes of PBS-BSA-Tween and incubated in drops of a rabbit anti-mouse IgG antibody (ICN Immunobiologicals) diluted to 25 ug/ml with PBS-BSA-Tween. After a one hour incubation in the secondary antibody, the grids were washed with PBS-BSA-Tween as described earlier and incubated in drops of 5 nm protein A-gold (Jannsen Life Sciences Products) or drops of 15 nm goat anti-rabbit IgG immunoglobulin-gold (Jannsen Life Sciences Products) diluted to 1/20 with PBS-BSA-Tween. The grids were incubated for one hour at room temperature and washed for 10 min in two changes of 0.1 M phosphate buffer, pH 7.3, with 0.01% Tween 20. The grids were then fixed for 15 min with 2.5% glutaraldehyde in 0.1 M phosphate buffer to stabilize the gold, rinsed with distilled water, dried, stained for 30 min with 2% uranyl acetate in 50% methanol, stained for 5 min with Reynold's lead citrate, carbon coated and examined with a JEOL 100CX electron microscope.

To test the specificity of the method, MAb 13E1 was substituted with MAb 6F2 which recognizes caveola-vesicle complexes of P. cynomolgi-infected erythrocytes. Other control sections were incubated with secondary antibody and gold probes or gold probes alone.

Results

In Vitro Development

At five days postinfection, cultured hepatocytes contained developing EE schizonts that ranged in size from small, uninucleated forms to large multinucleated parasites (Figs. 1-7). The most immature parasites contained a nucleus with numerous nuclear pores, mitochondria, scattered vacuolated areas, and random electron-dense granules. The parasitophorous vacuole membrane was closely apposed to the plasma membrane of the smallest EE parasites (Figs. 1, 2). In larger EE parasites, an electron-dense flocculent material was present in a thin layer between the closely apposed parasitophorous vacuole and schizont plasma membranes or within bump-like expansions of the parasitophorous vacuole space (Figs. 3, 4). Cytostomes with developing food vacuoles were occasionally observed in 5-day-old EE parasites (Fig. 5). Developing food vacuoles contained the same electron-dense flocculent material that was present in the parasitophorous vacuole space (Fig. 5). Large, multinucleated 5-day-old EE schizonts had numerous large peripheral vacuoles which contained electron-dense flocculent material (Fig. 6). These appeared to fuse with the plasma membrane of the developing schizont and release their contents into the parasitophorous vacuole space. The parasitophorous vacuole membrane surrounding these large schizonts had scattered patches of electron-dense material on its inner surface (Fig. 7). Mitochondria, endoplasmic reticulum, scattered electron-dense granules and occasional cytostomes were observed (Figs. 6, 7).

At eight days postinfection, cultured hepatocytes contained large EE schizonts with developing merozoites (Fig. 8). The schizont plasma membrane was deeply invaginated into the schizont cytoplasm, creating a highly contoured periphery with numerous interconnected "islands" of parasite cytoplasm. The schizont cytoplasm contained numerous nuclei, scattered electron-dense rhoptry precursors, mitochondria and electron-dense granules. The parasitophorous vacuole membrane was thickened and coated on its inner surface with fine, granular electron-dense material. The parasitophorous vacuole space was filled with electron-dense flocculent material.

Localization of CS Antigen

MAb 13E1 labeled the plasma membrane, parasitophorous vacuole membrane and some fine, electron-dense material in the parasitophorous vacuole space around uninucleated and larger multinucleated 5-day-old schizonts (Figs. 1-7). Density of labeling was most intense around the smallest EE schizonts (Fig. 1) and decreased significantly as the parasites increased in size (Fig. 8). Some scattered labeling occurred over the parasite cytoplasm, but it was not associated with specific structures (Fig. 1). The limiting membrane of developing food vacuoles was labeled by MAb 13E1, demonstrating its origin from the parasite plasma membrane; however, internalized food vacuoles containing electron-dense flocculent material were not observed (Fig. 5). The largest multinucleated 5-day-old schizonts were labeled sparsely by MAb 13E1 (Fig. 6, 7). Colloidal gold particles were restricted to patches of electron-dense material on the inner surface of the parasitophorous vacuole membrane, sparse amorphous, electron-dense material in the parasitophorous space and the schizont plasma membrane (Fig. 6, 7). Electron-dense

flocculent material which filled the large peripheral vacuoles and the parasitophorous vacuole space was not labeled (Fig. 7). Little label was associated with 8-day-old schizonts with developing merozoites (Fig. 8). Colloidal gold label was not associated with vesicles in the host cell cytoplasm or with the plasma membrane of infected host cells (Figs. 1-8).

Control sections incubated with the nonrelevant MAb, with secondary antibody and colloidal gold, or with colloidal gold alone were not labeled.

Discussion

Using the high resolution of immunoelectron microscopy, we were able to localize CS antigen on the plasma membrane, the parasitophorous vacuole membrane and some fine, electron-dense material in the parasitophorous vacuole space around immature 5-day-old P. cynomolgi EE schizonts. These observations confirm previous immunocytochemical studies of other malarial parasites that localized CS antigen within this intracellular compartment by light microscopy and document the persistance of this antigen during the earliest stages of EE development in in vitro cultures of P. cynomolgi.

Five-day-old EE parasites in our study were morphologically similar to in vivo schizonts of P. cynomolgi described previously. Sodeman et al. [11] described two types of vacuoles in 5-day-old P. cynomolgi schizonts - large, clear, membrane-bounded Type I vacuoles and smaller, electron-dense Type II vacuoles. The vacuolated areas we observed in 5-day-old schizonts may

correspond to Type I vacuoles of Sodeman et al.[11], while the electron-dense granules may correspond to the smaller Type II vacuoles; however, fixation with glutaraldehyde alone was not sufficient to preserve all structures clearly. Other features, including cytostomes, the thickened parasitophorous vacuole membrane and the presence of electron-dense flocculent material in peripheral vacuoles and in the parasitophorous vacuole space are similar to previous ultrastructural observations of malarial parasites.[12-16]

In contrast to recent studies of the EE stages of P. berghei, P. vivax, and P. falciparum, we observed a clear decrease in immunolabeling during in vitro EE development of P. cynomolgi. Labeling with MAb 13E1 was most dense around the smallest, most immature EE parasites and decreased significantly as the parasites grew and developed. Little label was associated with mature 8-day-old parasites. Our failure to detect persistent CS antigen in mature P. cynomolgi EE schizonts may represent differences in fixation and preservation of epitopes or differences in antibody affinity. Alternatively, these observations may indicate that P. cynomolgi sporozoites carry a finite amount of CS protein into host hepatocytes and stop synthesizing this antigen soon after invasion.

The potential function of persistent CS antigen on the parasitophorous vacuole membrane and surface of immature EE parasites is unknown. It may play some role in modifying this intracellular compartment - perhaps by preventing fusion of primary lysosomes with the parasitophorous vacuole membrane. Interestingly, CS antigen is apparently absent from the parasitophorous vacuole membrane around in vitro cultures of recently invaded

P. falciparum sporozoites.[6] *Plasmodium falciparum* sporozoites are unable to complete development in cultured hepatoma cells, suggesting that defective interaction of CS antigen with the parasitophorous vacuole membrane in these host cells may be a factor that restricts intracellular development.

Precisely how CS antigen is associated with the parasitophorous vacuole membrane is not clear. It may be inserted directly into the membrane as sporozoites enter hepatocytes or may simply slough from the sporozoite surface and adhere to the inner surface of the parasitophorous vacuole. Stewart and Vanderberg [17] and Suhrbier et al. [7] described trails of sloughed CS protein from extracellular and intracellular sporozoites and suggested that continuous synthesis and capping of the protein may be important in motility of invading sporozoites. These observations, as well as the patchy association of CS antigen with the parasitophorous vacuole membrane around older EE schizonts and the adherence of CS antigen onto the inner surface of the oocyst capsule during sporogony [18-21] suggest that CS antigen simply adheres to the parasitophorous vacuole membrane. Studies of this membrane using label-fracture techniques [22] may help to resolve this question.

We did not find evidence of CS antigen in vesicles in the host cell cytoplasm, on the surface of infected host cell or with structures analogous to the "extra-EE antigens" described by Hollingdale et al.. [4] The absence of such labeling is significant because it indicates that epitopes associated with the repeat region of the CS protein, an important vaccine candidate, may not be processed and expressed on the surface of infected hepatocytes as suggested by some authors. [23, 24] Consequently, sequestration of this

epitope within infected host cells may allow parasites to avoid the direct (i.e. cytotoxic) actions of CD8+ T lymphocytes. The absence of cellular infiltrates around intact host cells that contain developing EE schizonts *in vivo* supports this idea.[5, 25] Future ultrastructural studies that examine the distribution of additional CS epitopes, particularly T cell epitopes, in EE schizonts and host cells will provide important information about whether CS vaccines can be designed to be effective against EE parasites.

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Figure Legends

Figures 1 & 2. Sections of small 5-day-old P. cynomolgi EE schizonts that were incubated with MAb 13E1 to the P. cynomolgi CS protein, secondary antibody and 15 nm immunoglobulin-gold. 1. Small uninucleated trophozoite. Dense label (arrows) is associated with the surface of the parasite, completely obscuring the plasma membrane, parasitophorous vacuole membrane and parasitophorous vacuole space. Some scattered labeling is associated with the parasite cytoplasm. Internal structures include a nucleus (N) with numerous nuclear pores (arrowheads) and scattered vacuolated areas (V). x 26,000. 2. Larger uninucleated trophozoite. Label is associated with the surface of the parasite (P), with the parasitophorous vacuole membrane (PVM) and with fine granular material in the parasitophorous vacuole space. Internal structures include a nucleus (N), scattered mitochondria (Mi), and vacuolated areas (V). x 25,000.

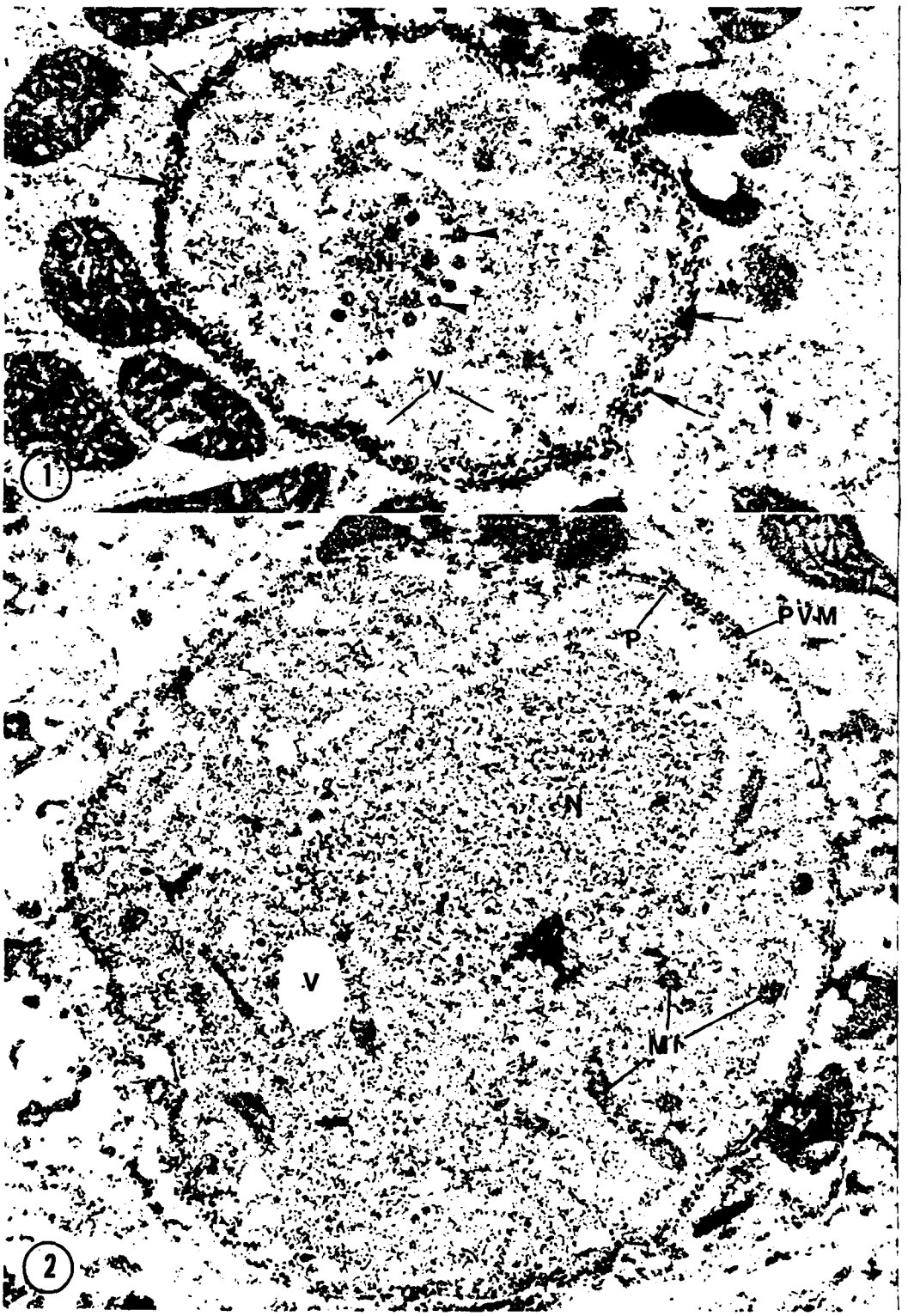
Figures 3-5. Sections of 5-day-old P. cynomolgi EE schizonts that were incubated with MAb 13E1 to the P. cynomolgi CS protein, secondary

antibody and 15 nm immunoglobulin-gold (Figs. 3, 5) or 5 nm protein A-gold (Fig. 4). 3. Large uninucleated trophozoite. Label is associated with the surface of the parasite (P), with the parasitophorous vacuole membrane (PVM) and with fine electron-dense material (EDM) in the parasitophorous vacuole space. Internal structures include a large, diffuse nucleus (N), scattered mitochondria (Mi), a few electron-dense granules (G) and vacuolated areas (V). A thin layer of electron-dense flocculent material (Fm) is present in the parasitophorous vacuole space. x 25,000. 4. Bump-like expansion of the parasitophorous vacuole membrane (PVM). The surface of the parasite (P) and the parasitophorous vacuole membrane are densely labeled by MAb 13E1. Some scattered label is associated with electron-dense flocculent material (Fm) in the parasitophorous vacuole space. The host cell cytoplasm and an adjacent host cell mitochondrion (Mi) are not labeled. x 56,000. 5. Cytostome (Cy) and developing food vacuole (Fv). Label (arrows) is associated with the surface of the parasite, with the indistinct parasitophorous vacuole membrane, and with fine electron-dense material in the parasitophorous vacuole space. Note the electron-dense flocculent material (Fm) in the developing food vacuole. x 44,800.

Figures 6 & 7. Sections of a multinucleated 5-day-old P. cynomolgi EE schizont that were incubated with MAb 13E1, secondary antibody and 15 nm immunoglobulin-gold. 6. Schizont contains numerous nuclei (N), scattered mitochondria (Mi) and some electron-dense granules

(G). Note large peripheral vacuoles (V) filled with electron-dense flocculent material. Only a few scattered gold particles (arrows) are associated with the surface of the parasite (P) and with the parasitophorous vacuole membrane (PVM). x 19,000. Inset. Small cytostome (Cy). Only a few gold particles (arrows) are associated with the surface of the parasite. x 36,400. 7. Higher magnification of Figure 6. Note peripheral vacuoles (V) filled with electron-dense flocculent material and sparse label (arrows) associated with the parasite surface (P) and with small patches of fine electron-dense material on the inner surface of the parasitophorous vacuole membrane (PVM). The host cell plasma membrane (Hm) is devoid of label. x 24,700.

Figure 8. Section of an 8-day-old P. cynomolgi EE schizont that was incubated with MAb 13E1, secondary antibody and 15 nm immunoglobulin-gold. The schizont plasma membrane is deeply invaginated into the schizont cytoplasm, creating a highly contoured periphery with numerous interconnected "islands" of parasite cytoplasm. Note nuclei (N) and electron-dense rhoptry precursors (R) of developing merozoites and the electron-dense flocculent material (Fm) which fills the expanded parasitophorous vacuole space. The parasitophorous vacuole membrane (PVM) is thickened and lined on its inner surface with a fine electron-dense material. Little label is associated with the schizont or parasitophorous vacuole membrane. x 23,250.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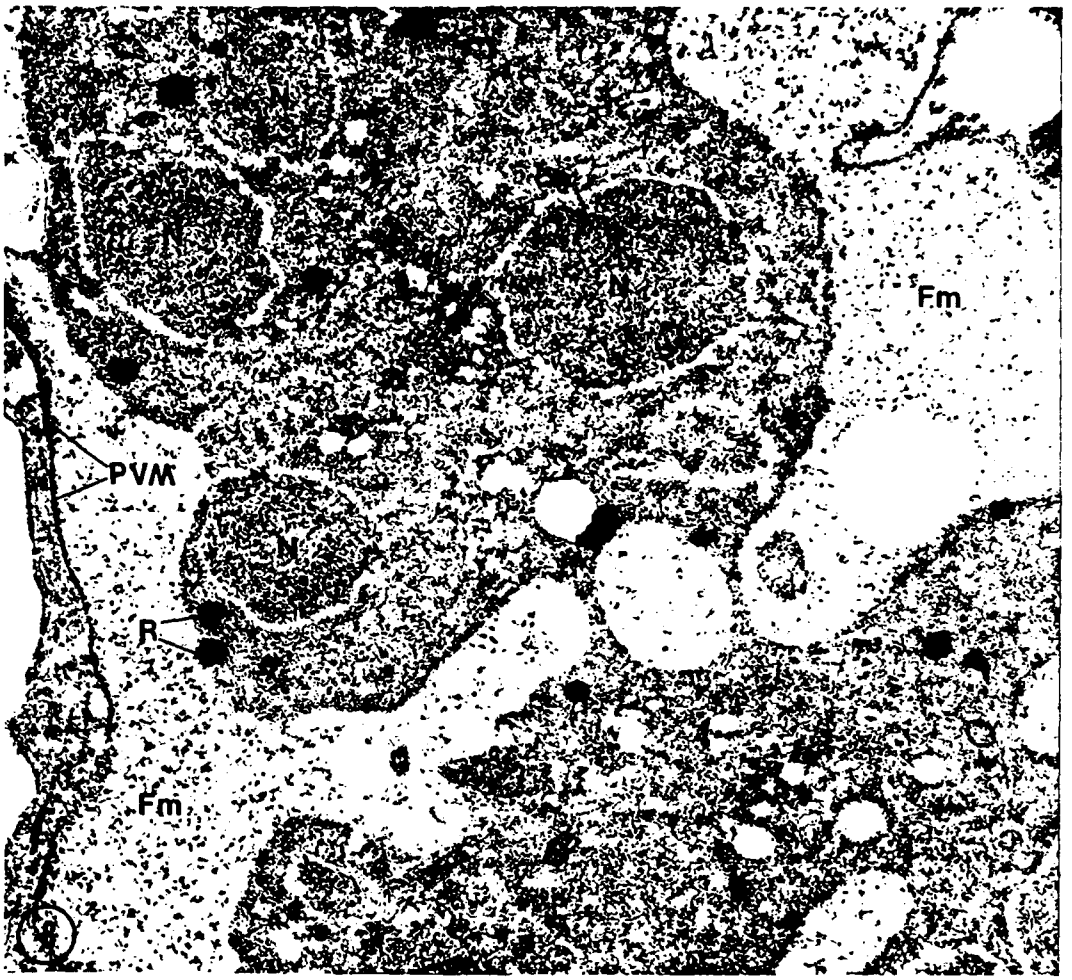
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Plasmodium malariae: Distribution of Circumsporozoite Protein in Midgut Oocysts and Salivary Gland Sporozoites

ABSTRACT

The distribution of the circumsporozoite protein within developing Plasmodium malariae oocysts and salivary gland sporozoites was examined by immunoelectron microscopy using protein A-gold and a monoclonal antibody specific for the CS protein of P. malariae. Gold particles were found along the capsule of immature oocysts but rarely within the cytoplasm. Gold label was detected on the inner surface of peripheral vacuoles during oocyst maturation and the plasma membrane of the sporoblast. Salivary gland sporozoites and budding sporozoites in mature oocysts were labeled uniformly on the outer surface of their plasma membranes. The surface of sporozoites that ruptured into midgut epithelial cells were entirely covered with gold particles. No label was seen on the surface of sporozoites which ruptured into the midgut lumen. In addition, a rabbit polyclonal antibody against repeat a region of P. brasilianum CS protein reacted with P. malariae sporozoites.

Index Descriptors and Abbreviations: Plasmodium malariae; Protozoa, parasitic; Malaria, human; Sporozoite; Protein, circumsporozoite; Plasmodium brasilianum; Malaria, simian; Microscopy, immunoelectron; Circumsporozoite (CS); Monoclonal antibody (MAb); Phosphate buffered saline (PBS); Bovine serum albumin (BSA).

INTRODUCTION

Malaria is transmitted to mammalian hosts by inoculation of sporozoites through the bite of mosquito vectors. The membranes of sporozoites are uniformly covered by a polypeptide called the CS protein (Yoshida et al. 1980). Several observations of CS proteins *in vitro* and *in vivo*, including an investigation demonstrating that MAbs against the CS proteins of a malaria parasite protect mice inoculated with viable sporozoites, suggest that these proteins are involved in the initial interaction of malarial sporozoites with target hepatocytes and are functionally important to the survival of sporozoites in the host (Potocnjak et al. 1980; Nussenzweig and Nussenzweig 1986). In addition, naturally acquired antibodies to CS proteins of *Plasmodium falciparum* have been demonstrated in serum from people living in areas in which malaria is endemic (Hoffman et al. 1986; Giudice et al. 1987; Campbell et al. 1987). These authors suggest that anti-CS protein antibody is important in reducing the prevalence of malaria with increasing age among persons in such areas and that vaccine-elicited antibody to the CS repeat region will contribute to the immune protection against malaria in humans. In spite of their importance, studies of the origin of CS proteins on the sporozoite surface are limited. Nagasawa et al. (In press) described by immunoelectron microscopy the distribution of CS proteins in developing oocysts of *P. ovale*. They demonstrated the presence of the CS protein in sporoblasts and oocyst sporozoites of *P. ovale* and suggested that precursors to the CS proteins are stored in the cytoplasm of sporoblasts prior to export to the sporoblast membrane.

In this study, we have used immunoelectron microscopy to determine the ultrastructural localization of CS proteins in midgut oocysts and salivary gland sporozoites of P. malariae, and to investigate the relationship of P. brasilianum and P. malariae CS proteins.

MATERIALS AND METHODS

A hybridoma secreting MAb against the CS protein of P. malariae was produced by previously described methodology (St. Groth and Scheidegger 1980). Briefly, the MAb 109-179.4 was produced by immunizing BALB/c female mice with 10^5 freshly dissected sporozoites of the Uganda I/CDC strain of P. malariae (Collins et al. 1984). The MAb produced a circumsporozoite precipitin reaction and recognized polypeptides of M_r 44,000, 49,000 and 56,000 of P. malariae sporozoites. A two-site enzyme linked immunosorbent assay using the MAb revealed the presence of a repeating epitope.

An antiserum was produced by immunizing a rabbit with the synthetic peptide, $(Asn\text{-}Ala\text{-}Ala\text{-}Gly)_5$, the amino acid sequence of which was derived from the DNA sequence of the immunodominant repeat of the CS protein of P. brasilianum (Lal et al., In press). The synthetic peptide was bound 1:1 (w/w) to keyhole limpet hemocyanin (KLH) with a final concentration of 0.25% glutaraldehyde. A rabbit received an injection of 1 mg peptide-KLH conjugate or glutaraldehyde treated KLH alone emulsified in complete Freund's adjuvant equally distributed between two intramuscular sites. Five weeks later a second injection of 1 mg of material was given in incomplete Freund's adjuvant; serum was collected two weeks later. The anti-peptide-KLH antiserum reacted in an indirect fluorescent antibody assay with both P. brasilianum and P. malariae sporozoites. The control anti-KLH antiserum did not react with the sporozoites.

Individual Anopheles stephensi mosquitoes were allowed to feed through artificial membranes on chimpanzee blood infected with P. malariae. The mosquitoes were collected 9-18 days after feeding and the midguts and

salivary glands were dissected into 1.25% glutaraldehyde in 0.1 M PBS containing 4% sucrose (pH 7.3) and fixed for 10 min at room temperature. Specimens were then washed twice in 0.1 M PBS containing 4% sucrose. They were dehydrated through a graded series of ethanol at progressively lower temperatures between 0°C and -20°C, and infiltrated with LR Gold resin containing 0.5% w/w benzoin methyl ether as an initiator. The midguts and salivary glands were transferred to gelatin capsules and polymerized for 30 hr at -20°C with UV light. Thin sections were cut with diamond knife, mounted on nickel grids (#400) and processed for immunolabeling.

Before labeling, the grids were floated on drops of a solution of saturated aqueus sodium metaperiodate (30 min at room temperature) to etch the surface of the resin and expose more antigenic sites. After washing with distilled water, sections were incubated with 5% nonfat dry milk in 0.1 M PBS containing 0.01% Tween 20 to block the nonspecific reaction. The sections were processed for immunocytochemical labeling by first floating the grids on drops of MAb 109-179.4 (mouse ascites diluted 1:200) in PBS-BSA (0.1 M PBS, pH 7.3, 1% BSA and 0.01% Tween 20) for 2 hr. All incubations were performed at room temperature. After washing with PBS-BSA, sections were incubated on drops of rabbit anti-mouse IgG (10 ug/ml in PBS-BSA) for 1 hr. The grids were rinsed thoroughly in PBS-BSA and then floated on a drop of protein A-gold particles (15 nm, Janssen, Belgium) diluted 1:10 in PBS-BSA, and incubated for 1 hr. The sections were washed in PBS-BSA and distilled water, and stained with 2% uranyl acetate in 50% methanol before examination in a JEOL 100 CX electron microscope.

The reactivity of the anti-*P. brasilianum* peptide antisera with *P. malariae* sporozoites was examined by the same procedure described above.

After incubation with rabbit immune serum diluted 1:100 in PBS-BSA, goat anti-rabbit IgG conjugated with gold particles (15 nm, Janssen, Belgium) was used instead of rabbit anti-mouse IgG and protein A-gold particles.

In order to evaluate the specificity of the labeling, the following controls were performed: 1) normal mouse ascitic fluid was used in place of MAb 109-179.4, 2) anti-KLH rabbit serum was used in place of anti-P. brasilianum peptide antisera, and 3) the preparations were incubated with protein A-gold alone.

RESULTS

Midgut oocysts from mosquitoes collected between 9 and 18 days after the infective blood meal ranged in maturity from young, immature forms to fully mature oocysts containing well developed sporozoites. At 18 days, sporozoites first appear in the salivary glands. Oocyst growth was similar to that of other species of Plasmodium (Aikawa and Sterling 1974). Briefly, the oocyst became increasingly vacuolated by the retraction of the oocyst plasma membrane from the oocyst capsule and by the formation of numerous cytoplasmic clefts. The clefts eventually coalesced to form a number of interconnected lobes that subdivided the oocyst cytoplasm into sporoblasts from which the sporozoites budded.

The binding of MAb 109-179.4 differed in immature and mature oocysts. Gold particles were not found in immature, non-vacuolated oocysts (Fig. 1, Insert). Specific label was first observed beneath the oocyst capsule of more mature, slightly vacuolated oocysts. The inner surface of peripheral vacuoles

and the oocyst capsule in more mature oocysts were covered entirely with gold particles (Fig. 1). A similar distribution was observed in highly vacuolated oocysts in which sporozoite budding had not begun. Fig. 2 shows that the peripheral vacuoles spread into the cytoplasm of the oocyst and that the inner surface was labeled uniformly with gold particles. In mature oocysts, MAb 109-179.4 bound primarily to the plasma membrane of sporoblasts that contained budding sporozoites (Fig. 3). In both oocyst sporozoites and salivary gland sporozoites, these plasma membranes were entirely covered in a dense, uniform fashion with gold particles (Fig. 4). Intracellular gold particles were also consistently associated with the cytoplasm of sporoblasts and sporozoites. Some label was associated with the micronemes and around the nucleus. Very few gold particles were found within nuclei, vacuoles and clefts in the cytoplasm. Some of the mature oocysts ruptured, liberating sporozoites into the midgut epithelium and into the midgut tract. The sporozoites in the epithelial cell were covered with gold particles on their surface. However, no gold particles were seen on the surface of the sporozoites which ruptured into the lumen of the midgut (Fig. 5). Nonspecific binding of protein A-gold was very low as determined by incubation with normal mouse ascitic fluid in place of primary antibody and by incubation with protein A-gold alone.

In addition, P. malariae salivary gland sporozoites were incubated with rabbit immune serum produced against a synthetic peptide $(NAAG)_5$ derived from the immunodominant repeat of the CS protein of P. brasilianum. The P. malariae sporozoites were labeled uniformly with gold particles on their surface (Fig. 6).

DISCUSSION

Sporozoites obtained from midgut oocysts and salivary glands are morphologically very similar, but are biologically quite different. Salivary gland sporozoites appear to be more infective, and can be used to vaccinate mammalian hosts (Vanderberg et al. 1972; Vanderberg 1975). Animals immunized with salivary gland sporozoites can acquire protective immunity to sporozoite-induced infection. Antibodies from sporozoite-immunized animals react with a single immunodominant protein that covers the surface of the sporozoites, that is, the circumsporozoite protein (Zavala et al. 1983).

We have previously shown that the CS protein is present in oocyst sporozoites and sporoblasts of P. ovale (Nagasawa et al., In press). In this study, we used immunoelectron microscopy with protein A-gold and MAb 109-179.4 which reacts with CS proteins of P. malariae to observe in detail the distribution of CS proteins in developing oocysts and salivary gland sporozoites. We have demonstrated that the antigen recognized by MAb 109-179.4 was found on the developing oocysts and was consistently present on salivary gland sporozoites. Gold particles were first observed beneath the oocyst capsule of slightly vacuolated oocysts. As the peripheral vacuoles spread, the density of gold particles increased. When the sporoblast was formed by vacuolation and retraction, the plasma membranes of the sporoblast were covered entirely with gold particles. The binding of the gold particles to the surface of oocyst sporozoites was observed at the time of budding from sporoblast. The results of this study suggest that CS proteins of salivary gland P. malariae sporozoites are probably derived from the oocyst capsule.

Recently, Schwartz et al. (1986) described the sporogonic development of P. vivax in An. stephensi using a MAb-based enzyme-linked immunosorbent assay for the CS protein. They suggested that immunologically recognizable CS protein was present within the oocyst before sporozoites are noted, but that the antigen was not in the salivary glands until sporozoites were seen. This data strongly supports our findings that gold particles were found in immature oocysts in which sporozoite budding had not begun, indicating that CS proteins were present within the oocyst before sporozoites were noted.

The process of vacuolation in oocysts is achieved by two simultaneous but different mechanisms, namely, formation by peripheral and cytoplasmic vacuoles (Sinden and Strong, 1978). Our data have shown these differences by the distribution of gold particles. Since gold particles were found only on the inner surface of peripheral vacuoles, sporoblast formation is certainly preceded by peripheral subcapsular vacuolation, and coalescence of these vacuoles which subdivide the oocyst cytoplasm.

The present findings indicated specific binding of MAb 109-179.4 to the surface of sporozoites which ruptured into the cytoplasm of midgut epithelial cells. However, sporozoites which ruptured into the midgut lumen demonstrated no labeling on the surface, whereas intracellular gold particles were frequently found to be associated with micronemes. This finding may be explained by enzymatic digestion of the surface protein of sporozoites. Fine et al. (1984) reported that limited proteolysis of P. knowlesi sporozoites, prior to cryosectioning and incubation with MAb and the protein A-gold label, totally eliminated binding of label to the outer plasma membrane. In contrast, distribution of label on the inner pellicular

membranes, rhoptries, and micronemes of trypsinized sporozoites was relatively unaltered.

P. brasilianum, the quartan malaria parasite of Neotropical monkeys, is thought to have arisen from the natural infection of New World monkeys with the human malaria parasite P. malariae during the European conquest of Latin America (Coatney et al. 1971). Cross reactions between P. malariae and P. brasilianum antigens using antibodies have been well documented; Collins et al. (1966) demonstrated the presence of cross-reacting antibodies between P. malariae and P. brasilianum in human serum. In addition, MAbs produced against the CS protein of P. malariae cross-react with P. brasilianum sporozoites (Cochrane et al. 1984) and those MAbs abolish the infectivity of P. brasilianum sporozoites (Cochrane et al. 1985). In this study we used a rabbit antiserum generated against an immunodominant repeat, $(NAAG)_5$, of the CS protein of P. brasilianum to establish the degree of relatedness of the cross-reactivity between P. malariae and P. brasilianum CS proteins. The reactivity of the rabbit antisera with the P. malariae salivary gland sporozoites demonstrates a close similarity between the CS proteins of P. malariae and P. brasilianum. This cross-reaction is of interest because of the potential usefulness of non-human models of malaria for immunologic and vaccine studies. Consequently, a better understanding of the biology of CS proteins may be of considerable scientific and practical interest.

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FIGURE LEGENDS

Fig. 1 Young oocyst of Plasmodium malariae in the midgut of Anopheles stephensi. The inner surface of peripheral vacuoles (PV) and oocyst capsule (OC) are covered entirely with gold particles. Bar indicates 0.5 um. Inset: High magnification showing the non-vacuolated oocyst. ME, midgut epithelium. Bar indicates 0.5 um.

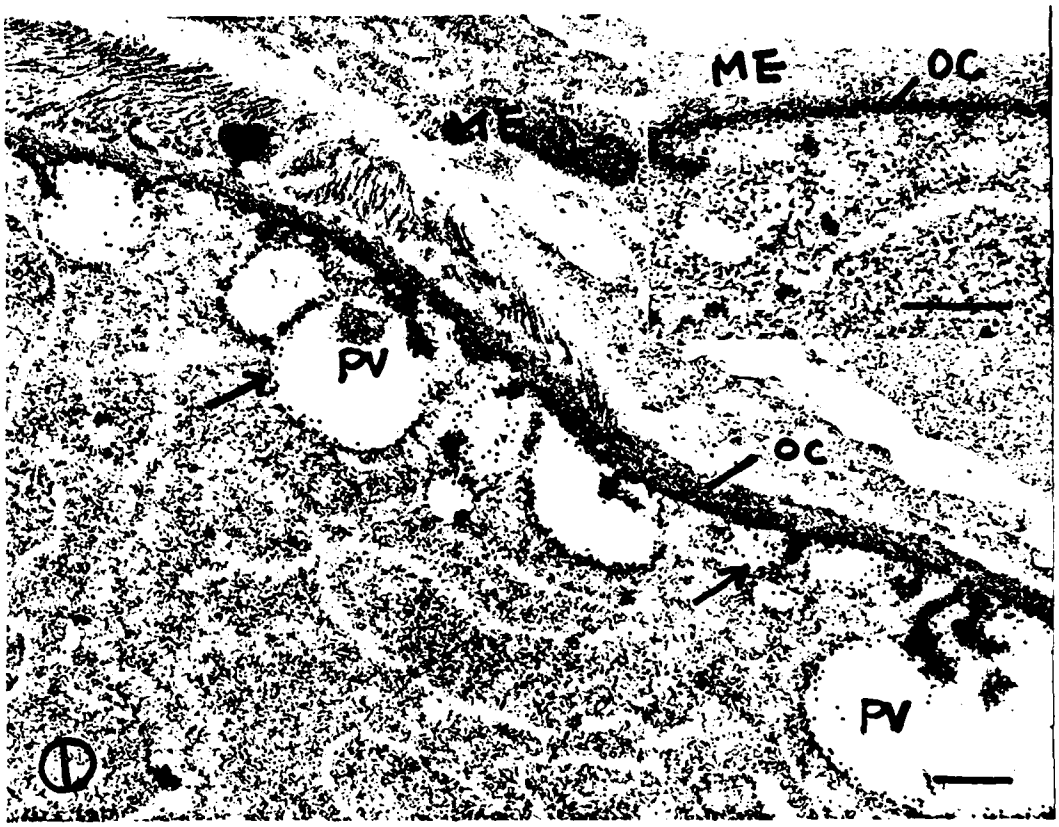
Fig. 2. Immature, highly vacuolated Plasmodium malariae oocyst in which sporozoite budding has not begun. The inner surface of peripheral vacuoles (PV) and oocyst capsule (OC) is labeled uniformly with gold particles. ME, midgut epithelium; N, nucleus. Bar indicates 1 um.

Fig. 3. Mature oocyst of Plasmodium malariae. Gold particles are primarily bound to the plasma membranes of sporoblast (Sb) and oocyst sporozoite (OS). Very few gold particles were found over the nucleus and cytoplasmic vacuole (CV). Bar indicates 0.5 um.

Fig. 4. Plasmodium malariae sporozoite in the secretory cavity (SC) of lateral salivary gland lobe of Anopheles slephensi. Gold particles cover the plasma membranes of salivary gland sporozoites (SS) continuously. Bar indicates 0.5 um.

Fig. 5. Mature ruptured oocyst of Plasmodium malariae liberating sporozoites into the lumen of midgut. Very few gold particles are seen on the surface of the sporozoites. V, villi. Bar indicates 1 um.

Fig. 6. Plasmodium malariae salivary gland sporozoite reacted with rabbit anti-Plasmodium brasilianum peptide antibody, followed by goat anti-rabbit IgG conjugated with gold particles. The gold particles are bound to the surface of plasma membrane and to the micronemes (M). SC, secretory cavity. Bar indicates 0.5 um.



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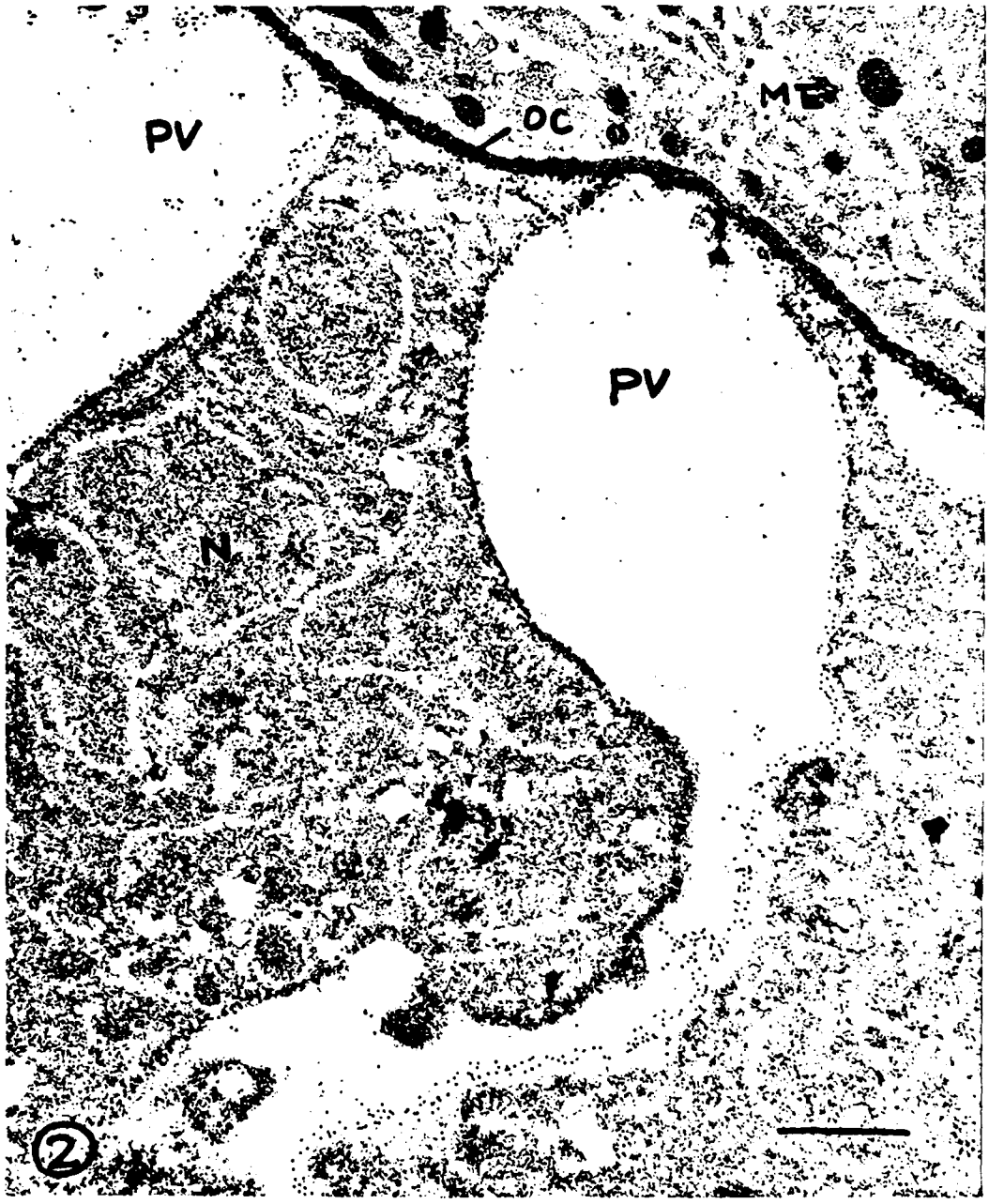

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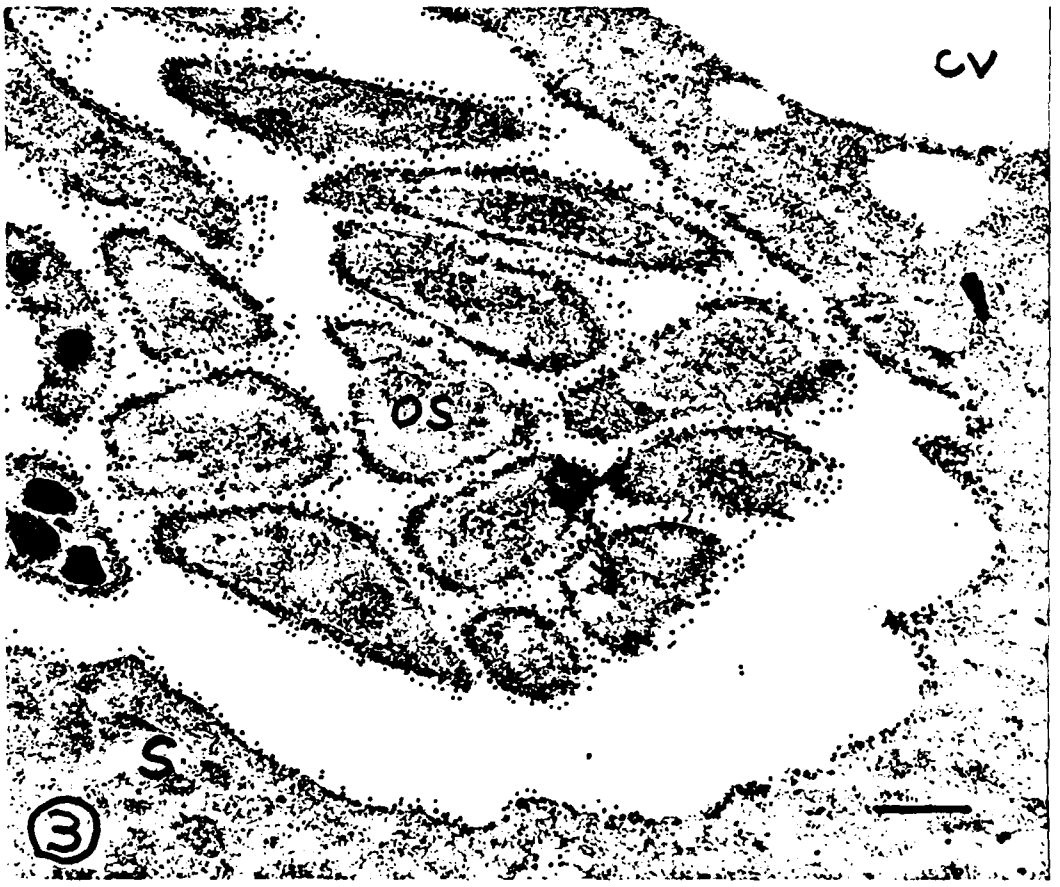

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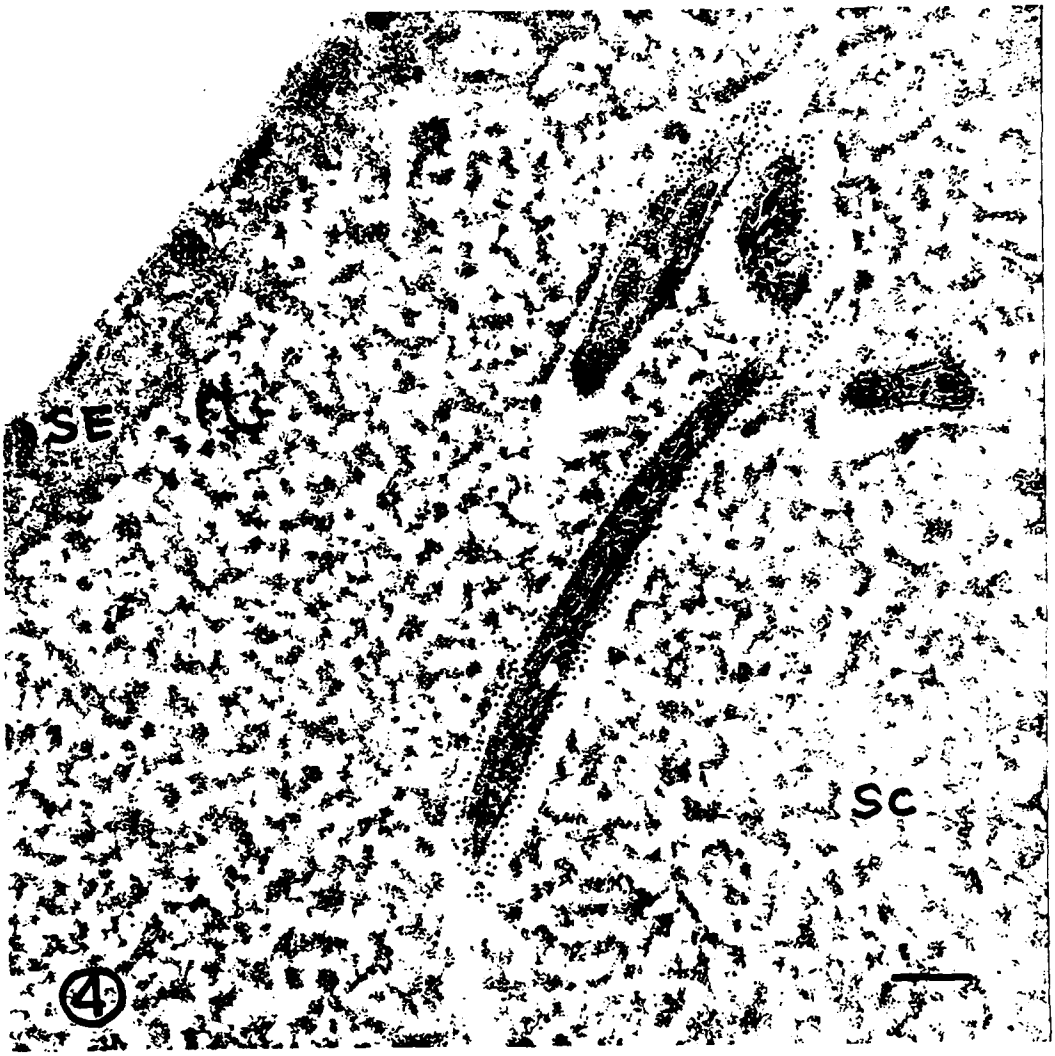

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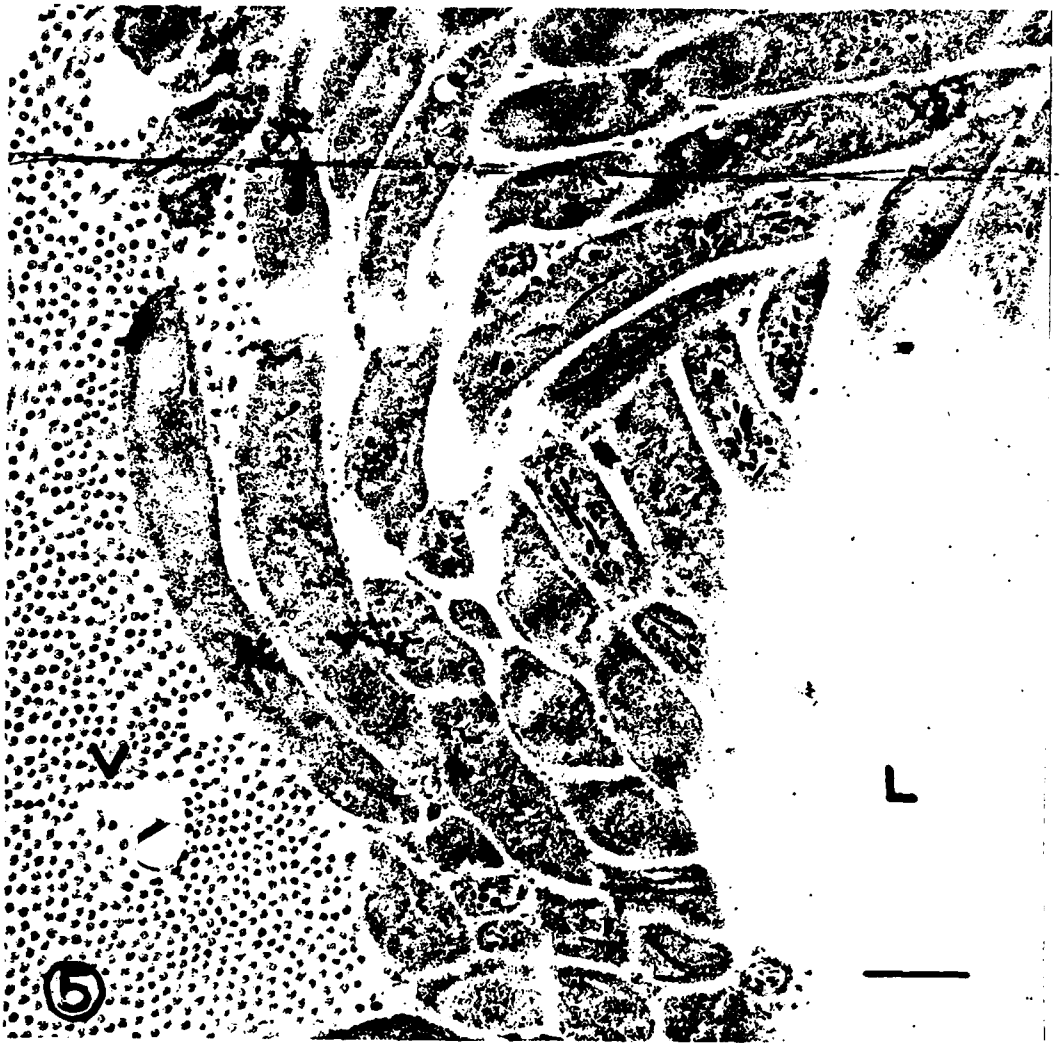

Fig. 5

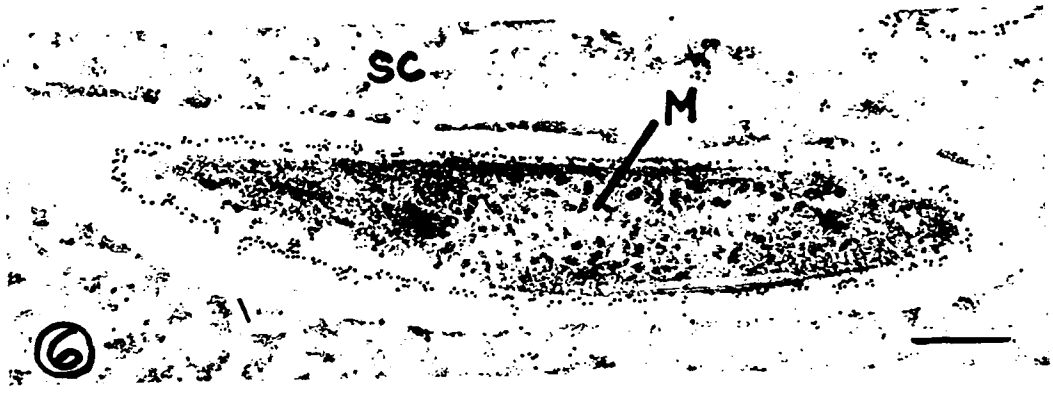

Fig. 6

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